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PRINCIPAL INVESTIGATOR: Russell Szmulewitz

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14. ABSTRACT

The purpose of the award was to provide Dr. Szmulewitz with training in basic and translational prostate cancer (PC) research. The scientific focus is PC metastasis modeling and modulation of metastatic colonization (the specific outgrowth of microscopic disseminated cancer cells to overt macroscopic metastases) through the prostate cancer metastasis suppressor JNKK1/MKK4. In addition the scope of the award also includes key training opportunities. Over the second year of this award Dr. Szmulewitz continues to utilize resources within the cancer community at the University of Chicago. He continues to take coursework critical to his career development as a physician-scientist and is now an integrated member of the University of Chicago Comprehensive Cancer Center. Dr. Szmulewitz has completed the work to date outlined within the Statement of Work. The scientific aim of the award was to show that the metastasis suppressor JNKK1/MKK4 when ectopically expressed in Dunning PC cells, impairs metastatic colonization through alteration in proliferation at the secondary site where the kinase is activated. Studies have been completed and analyzed within Aim 1 supporting that JNKK1/MKK4 mediated metastasis suppression is imparted through a transient delay in cell-cycle progression. Furthermore, studies within Aim 2 have shown that this transient delay in metastasis formation is a delay in proliferation within the first 14 days post dissemination. Eventual outgrowth of metastases is concordant with a loss in JNKK1/MKK4 kinase activity within the overt metastases, signifying an initial activation of the kinase, leading to suppression of colonization after dissemination, and eventual cellular adaptation through loss of kinase activity. This work was recently published in The International Journal of Cancer.

15. SUBJECT TERMS

Prostate Cancer, Metastatic colonization, Metastasis, Cell-cycle, JNKK1/MKK4

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Table of Contents

I. Introduction

The following report is a documentation of relevant progress made on Department of Defense Physician Research Training Award (PRTA) within the Prostate Cancer program entitled, "Control of Metastatic Colonization in Prostate Cancer: the Functional Mechanism of Metastasis Suppression by JNKK1/MKK4" (award # W81XWH-09-1-0415, P.I. Russell Szmulewitz, MD) over the second year of this award. The purpose of the award was to provide the principle investigator with training in basic and translational prostate cancer research through a focused training plan and specific research project.

I have the stated goal of becoming a physician-scientist with established expertise in the evolution of prostate cancer metastasis. As such, the scientific focus of this training award is metastatic prostate cancer modeling and modulation of metastatic colonization (the specific outgrowth of microscopic disseminated cancer cells to overt macroscopic metastases) through the prostate cancer metastasis suppressor JNKK1/MKK4 (MKK4). In addition to discrete scientific aims as outlined in the proposal and statement of work (SOW), the scope of the award also includes key training opportunities.

II. Body

This progress report will be organized into two primary sections (Training Program and Research Project) and will be further organized to specifically address the accomplishments stated within the proposal and SOW.

Training Program

Didactic Training: The didactic training program put forth in this PRTA focus on utilizing the resources available at the University of Chicago along with mentors both at the University of Chicago and externally. According to the statement of work, I was to attend certain courses. As stated in last years report, my clinical duties prohibit me from taking 100% of the lectures nor take the courses for credit.

1. As per my SOW, I attended CABI 39000 (Intro to Experimental Cancer Biology) throughout the entire year. This is a journal club based cancer biology graduate course that runs the entire year.
2. The other cancer biology course I was to take during this year was CABI 31500 (Fronteirs in Cancer Biology). I was named by my mentor Dr. Rinker-Schaeffer (course director) as an instructor for this course. I thus completed this course as a faculty member.
3. I was to take two health studies courses this year in biostatistics (HSTD 31200- Intro to biostatistics and HSTD 32700 Biostatistical methods). HSTD 31200 was not offered this year and thus this was an ***unanticipated difficulty***. HSTD31200 is a prerequisite for HSTD 32700. In lieu of the formal coursework, which was not accessible to me this year, I have met with Theodore Karrison, PhD and director of the biostatistics laboratory, and Masha Kocherginsky, PhD- faculty within the

biostatistics laboratory at the University of Chicago regularly (monthly) to train on the biostatistical techniques relevant to my work. HSTD 31200 will be offered this year and will be taken in the summer 2012.

Mentorship: I continue to work closely with my research mentors for my basic science and clinical research. Specifically, I meet with Dr. Rinker-Schaeffer and Dr. Stadler at a minimum bi-weekly to address the specific details of his ongoing research. I also continue to work with the other mentors described in his training proposal at the regular intervals described in my proposal.

Other Opportunities: It is clear that didactic training and research mentorship are key components to my training plan. In addition, there are other training opportunities that I have capitalized on over the first year of his PRTA award as suggested by my mentors. I attended national and international research conferences (AACR national meetings, DOD IMPaCT meeting, ASCO Genitourinary Symposium). I am a member of the University of Chicago Comprehensive Cancer center and am integrated within bioscience community at the University of Chicago and am now a faculty instructor in two graduate level Cancer Biology courses. In addition, I was named to the Cancer Center's clinical trial scientific review committee.

Research Project

Title: Control of Metastatic Colonization in Prostate Cancer: the Functional Mechanism of Metastasis Suppression by JNKK1/MKK4

Introduction:

The development of overt prostate cancer metastases is a complex and dynamic process, whose details remain unclear. Metastatic colonization [defined above] is a critical, rate-limiting step in metastasis formation. Experimental evidence also indicates that it is a potential target for anti-metastatic therapies. JNKK1/MKK4 has been shown to inhibit the process of metastatic colonization in an experimental model of prostate cancer metastasis. Nonetheless, there were several key questions that my proposal sought to clarify including: 1) How does JNKK1/MKK4 impart metastasis suppression at the molecular and cellular level? 2) Are these findings seen in other prostate cancer cell lines, or is the findings of metastasis suppression unique to the AT6.1 cell line studies previously? 3) How do prostate cancer cells overcome metastasis suppression?

The research project proposed within this PRTA was divided into two specific aims, with predictions/sub-aims to accomplish within each aim. This portion of the report will be organized by specific aim, and according to the work within defined in the SOW.

Aim 1: *To test the hypothesis that JNKK1/MKK4 inhibits metastasis formation by impaired proliferation of disseminated prostate cancer cells through a reversible induction of cell cycle inhibitor proteins.*

The majority of the work within this aim was completed in year one of this award and was summarized in last years report. According to the SOW the tasks within this Aim were to complete the *in vivo* proliferation experiments and to perform biostatistical analysis of the data. One experiment that was attempted was to utilize *mCherry* fluorescently labeled cancer cells incubated with an alternative fluorescently labeled microbead to image cancer cell colonization/proliferation within the lung. The stable *mCherry* expressing cells lines were established. However at early time points post tail vein injection, they are not visible at the surface of the lung (or by IHC-see last year's report). Thus, this method was not feasible for use within this project. The flow cytometry based studies described last year were used as an alternative. A full biostatistics analysis has been completed, in concordance with the statement of work, and the results demonstrating completion of Aim 1 have been successfully published (Figures 2,3,4,6-Szmulewitz et al., *International Journal of Cancer*, 2011; attached as an appendix).

Aim 2: *To test the hypothesis that JNKK1/MKK4 is activated at the metastatic site early after dissemination, leading to metastasis suppression, but that over time, JNKK1/MKK4 is no longer activated, and ceases to suppress metastases.*

According to the statement of work, the following work was to be accomplished within year 2 of this ward.

Prediction 1: Cell lines derived from overt AT6.1-JNKK1/MKK4 metastases will still express the functional HA-JNKK1/MKK4 construct, and will still actively suppress metastases when reinjected into mice.

Approach 2: In vivo characterization of metastasis derived cell lines- Experimental metastasis assays will be performed with metastasis derived cell lines and analysis of metastasis suppression at 28 days post injection (DPI) when compared to parental AT6.1-JNKK1/MKK4 and AT6.1-Vector cell lines.

This work has been successfully completed and published (Szmulewitz et al., *International Journal of Cancer*, 2011; attached as an appendix). Figure 5d demonstrates that *in vivo*, metastasis derived AT6.1-JNKK1/MKK4 cell lines remain suppressed for metastasis when reinjected via tail vein into immunocompromised mice. Compared to parental vector expressing cells, there was a significant reduction in surface metastases seen at 28 DPI ($p<0.0001$), whereas there was no change compared to parental AT6.1-JNKK1/MKK4 surface metastases.

Prediction 2: JNKK1/MKK4 will be activated early during the time course of metastatic colonization within disseminated cells but not within overt metastases. *I anticipate that Aim II, prediction 2 will be completed in the final 24 months.*

The work to be completed within year 2 within this prediction of Aim 2 include:

1. Optimize *in vitro* kinase assay on fresh lung tissue after mice injected via tail vein with various numbers anisomycin stimulated JNKK1/MKK4-AT6.1 cells, and establish limit of detection. This work was completed. As depicted in Figure 1 below, using AT6.1-JNKK1/MKK4 cells stimulated *in vitro*, injected via tail vein, the limits of detection for the *ex vivo* kinase assay is approximately $1x10^5$ JNKK1/MKK4 kinase active cells.

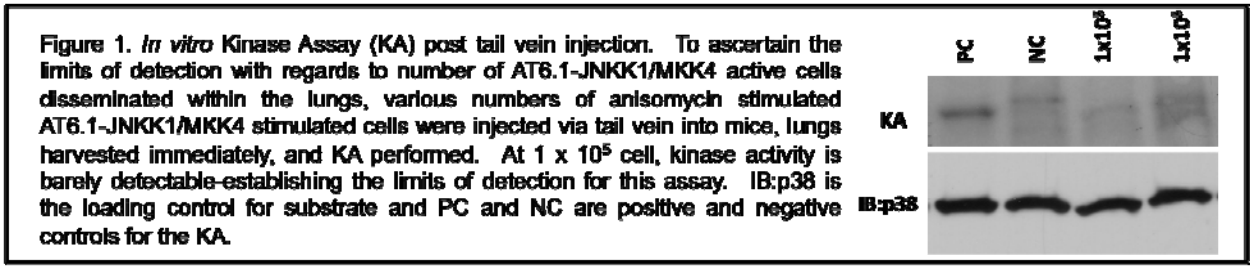

Figure 1. *In vitro* Kinase Assay (KA) post tail vein injection. To ascertain the limits of detection with regards to number of AT6.1-JNKK1/MKK4 active cells disseminated within the lungs, various numbers of anisomycin stimulated AT6.1-JNKK1/MKK4 stimulated cells were injected via tail vein into mice, lungs harvested immediately, and KA performed. At 1 x 10^5 cell, kinase activity is barely detectable-establishing the limits of detection for this assay. IB:p38 is the loading control for substrate and PC and NC are positive and negative controls for the KA.

2. Initiate *ex vivo* characterization of disseminated AT6.1-JNKK1/MKK4 parental cells- Perform functional kinase assays for 1, 7, 14, 21, 28, and 42 DPI experimental end points. This work has also been completed to the best of my ability. At endpoint (42 DPI), overt metastases do not have active JNKK1/MKK4 kinase. This finding was published (Figure 5C, Szmulewitz et al., *International Journal of Cancer*, 2011; attached as an appendix). At earlier time points, repeated *ex vivo* kinase assays were preformed on lung tissue lysates. Unfortunately, the limits of detection established above, indicate that at low cell numbers, this assay will not be sensitive. As shown in Figure 2, in addition to inadequate sensitivity of the KA on whole lung tissues, there is also a variable degree of background noise. At 8 DPI, in one sample below the KA is positive, all others are negative or confounded by background noise. This was repeated multiple times, with various methodological alterations, all with similar results. At early time points post injection, the KA is insufficient to refute or support the hypothesis.

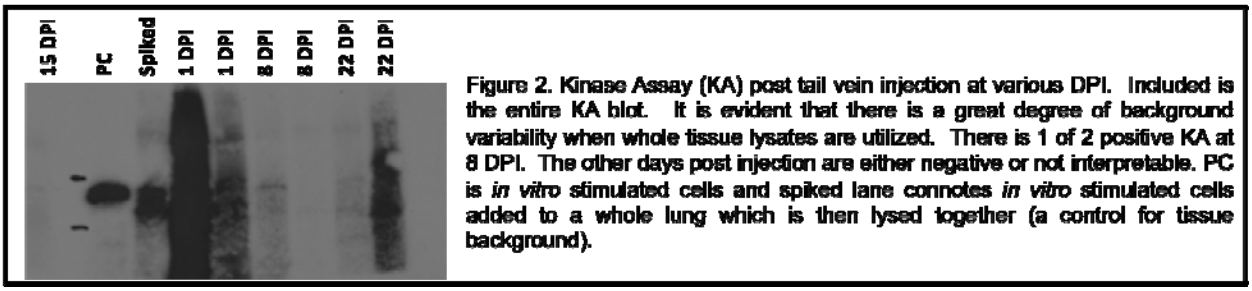

Figure 2. Kinase Assay (KA) post tail vein injection at various DPI. Included is the entire KA blot. It is evident that there is a great degree of background variability when whole tissue lysates are utilized. There is 1 of 2 positive KA at 8 DPI. The other days post injection are either negative or not interpretable. PC is *in vitro* stimulated cells and spiked lane connotes *in vitro* stimulated cells added to a whole lung which is then lysed together (a control for tissue background).

As an alternative, an analysis of the metastatic kinetics was undertaken. To show that there is a transient activation of the kinase, suppressing proliferation for a limited period

post injection, surface metastases were examined at various time points post injection of AT6.1 vector and JNKK1/MKK4 expressing cells. A non-linear regression analysis was performed demonstrating a transient ~14 day delay in metastatic colonization after dissemination of the cancer cells imparted by JNKK1/MKK4 expression (Figure 5A, Szmulewitz et al., *International Journal of Cancer*, 2011; attached as an appendix). These data, along with the lack of kinase activity in overt AT6.1-JNKK1/MKK4 metastases and the data from *prediction 1*, supports that early, transient JNKK1/MKK4 kinase activity is the critical mediator of metastasis suppression.

III. **Key Research Accomplishments**

The following research accomplishments have been completed during this period.

Aim 1.

- AT6.1 prostate cancer cell lines stably expressing the mCherry fluorescent protein in addition to JNKK1/MKK4 or empty vector were developed and utilized.
- Metastasis suppression was found to be associated with growth inhibition and cell cycle arrest in G1, not apoptosis, consistent with the stated hypothesis.
- Full biostatistic analysis of data generated during years 1, 2 was conducted and

Aim 2.

- Overt AT6.1-JNKK1/MKK4 metastases were found to lack kinase activity
- AT6.1-JNKK1/MKK4 metastasis derived cell lines (established from overt metastases) were found to retain metastasis suppressive ability *in vivo* when reinjected.
- A 14 day delay in proliferation is imparted by active JNKK1/MKK4 kinase within AT6.1 cells, after which, metastatic colonization parallels AT6.1-Vector cells.

IV. **Reportable Outcomes**

The following are reportable outcomes that have occurred during the 2nd year of my funding (9/1/10-8/31/11).

Poster Presentation: "MKK4 Suppresses Metastatic Colonization by Multiple Highly Metastatic Prostate Cancer Cell Lines Through a Transient Impairment in Cell Cycle Progression". DOD IMPaCT meeting March 2011.

Faculty promotion: As a direct consequence of the PRTA award and its support, I have achieved promotion to Assistant Professor of Medicine within the Department of Medicine at the

University of Chicago.

Publication: As noted above, the work supported to date by my PRTA has been published this year. Szmulewitz RZ, Clark R, Lotan T, Otto K, Veneris J, Macleod K, Rinker-Schaeffer C. "MKK4 Suppresses Metastatic Colonization by Multiple Highly Metastatic Prostate Cancer Cell Lines Through a Transient Impairment in Cell Cycle Progression," *International Journal of Cancer. Feb 2011 e-pub ahead of print.*

In addition, I was a co-author on an invited review article.

Shoushtari A, Szmulewitz RZ, Rinker-Schaefer. "Metastasis suppressor genes: Lost in translation." *Nature Reviews Clinical Oncology*. 2011. 8(6): 633-42.

Grant Awarded: As a consequence of the protected time and research training afforded by the PRTA, I was able to collaborate with another faculty at the University of Chicago to pursue a novel research hypothesis in advanced prostate cancer. I was awarded the American Cancer Society, Institutional Research Grant for the pilot project, "Serum and glucocorticoid-regulated kinase1 (SGK1) and the development of castration resistant prostate cancer (CRPC)". 12/1/10-ongoing.

V. Conclusion

During the second year of the DOD PRTA in the Prostate Cancer Research Program I have made continued significant progress in my training and research consistent with my statement of work for this award. The Aims of the research plan and goals of the training program are appropriately moving forward. The work has been published within the well respected journal *The International Journal of Cancer*. Unanticipated difficulties within my training program and research program have been encountered (detailed above) and necessary adjustments are being employed. In sum, the training and research supported within the PRTA has made a tremendous impact on my career as an early career physician scientist. I have achieved faculty appointment at the University of Chicago and am now pursuing independent research opportunities built on the foundation provided by the PRTA. Furthermore, the now published research is a significant contribution to the prostate cancer field. It supports the notion that metastatic prostate cancer is a dynamic process in which disseminated cancer cells can become transiently quiescent through interactions with their microenvironment that regulate intracellular processes controlling cancer cell proliferation. Future studies along this line of inquiry will specifically test the role of JNKK1/MKK4 regulation of prostate cancer metastasis of human prostate cancer cells within the bone microenvironment. A revised statement of work will be submitted for this future work.

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MKK4 suppresses metastatic colonization by multiple highly metastatic prostate cancer cell lines through a transient impairment in cell cycle progression

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Metastatic dissemination in prostate cancer is often early, but not all cancer cells form clinical metastases. Map kinase kinase 4 (MKK4) suppresses metastasis in a preclinical prostate cancer model. We hypothesize that MKK4 will specifically inhibit metastatic colonization through impaired proliferation. Three highly metastatic rat prostate cancer cell lines (AT6.1, Mat-Lu and AT3.1) were employed. Stably over-expressing HA-MKK4 or vector control lines were injected into immunocompromised mice. These experiments validated that HA-MKK4 specifically affects metastatic colonization and increases survival. Median survival (days) with HA-MKK4 *vs*. vector was 42 *vs*. 28 ($p < 0.0001$) for AT6.1, 25 *vs*. 19 ($p < 0.0001$) for Mat-Lu and 27 *vs*. 20 ($p < 0.0001$) for AT3.1. HA-MKK4 suppresses colonization within 14 days post dissemination, after which exponential proliferation resumes. Although overt metastases retain HA-MKK4, it is inactive within these lesions. Nonetheless, metastasis-derived cell lines were shown to retain functional HA-MKK4 and like their parental HA-MKK4 line are suppressed for experimental metastasis formation *in vivo*. Disseminated AT6.1-HA-MKK4 cells were analyzed and were found to have an alteration in cell cycle. Specifically, there was an accumulation of cells in G1-phase ($p = 0.024$) and decrease in S-phase ($p = 0.037$) compared with vector. In multiple prostate cancer lines, HA-MKK4 suppresses an early step in metastatic colonization. These data support a model in which MKK4 activation at the metastatic site causes a cell-cycle arrest, which is eventually overcome despite presence of functional HA-MKK4. Further studies will specifically interrogate the regulation of MKK4 activation within the metastatic microenvironment and the down-stream molecular events critical for metastasis suppression.

This year, it is estimated that 569,490 Americans will die from cancer; most of them from metastatic disease.[1] There is growing recognition that cancer cells disseminate from the primary tumor early in the natural history of many cancers. For example, although more than 90% of prostate cancers are considered to be localized at the time of diagnosis, after definitive local therapy, 20–40% of patients will develop clinically detectable recurrent disease.[2] Accumulated evidence supports that prostate cancer cells disseminate to metastatic sites early in the course of disease and can remain undetected for extended periods of time.[3–5] Such findings have sparked intense interest in understanding metastatic dormancy in an effort to prevent or control late-arising metastases.[6–10] The factors that control the survival and subsequent growth of disseminated cells are largely unknown. Thus, there is a crucial need to discern cellular and molecular mechanisms that regulate metastatic colonization, which is the progressive growth of disseminated cells at metastatic sites.

There is increasing use of metastasis suppressor proteins as tools to query the clinically tractable process of metastatic colonization. Our efforts have focused on the metastasis-suppressive effect of the c-Jun NH_2-terminal kinase activating kinase 1/mitogen-activated protein kinase (MAPK) kinase 4 (JNKK1/MKK4; hereafter referred to as MKK4), a key member of the stress-activated protein kinase (SAPK) signaling cascade. Multiple studies support a role for MKK4 in the suppression of metastatic growth in ovarian as well as prostate cancer.[11–14] Ectopic expression of MKK4 in AT6.1 Dunning prostate cancer cells reduces spontaneous metastases formation by ~80% ($p < 0.0001$) and increases survival by

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~60% ($p < 0.0001$) in immunocompromised mice and syngeneic rats.[15,16] Preliminary studies showed that MKK4 is not active within the primary tumor but becomes activated after cells lodge within the lung. These findings raise important questions: can MKK4 directly impair the ability of highly metastatic cells to colonize target sites? If so, what is the magnitude and duration of this suppression? Can MKK4-expressing cells become resistant to or adapt to the effects of MKK4? Activation of MKK4 and its downstream targets p38 and JNK can lead to various cellular sequelae including cell cycle arrest and apoptosis.[17] In the SKOV3.ip ovarian cancer experimental metastasis model, ectopic expression of MKK4 leads to inhibition of proliferation, possibly mediated by the cell cycle inhibitor p21.[13] The molecular mechanism of metastasis suppression in the prostate cancer model is not known.

Experiments detailed herein were designed to test the hypothesis that ectopic expression of MKK4 specifically suppresses metastatic colonization by highly metastatic variants of the Dunning model of rat prostatic cancers. The Dunning model has been used successfully for many years in basic and translational studies of prostate cancer. It originated in a spontaneous rat prostate adenocarcinoma and is comprised of multiple distinct well-characterized cell lines (Fig. 1). This model is especially useful in studies of metastasis as many Dunning cell lines form reproducible numbers of spontaneous metastatic lesions.[18,19] In particular, the Dunning model has proven to be a powerful tool in the identification and evaluation of metastasis suppressor genes.[15,16,20–31]

Using robust *in vivo* studies, we show that MKK4 significantly reduces the ability of highly metastatic, Dunning Mat-Lu, AT3.1 and AT6.1 cells to colonize target organs through a transient cell cycle arrest. Our data also show that contrary to conventional wisdom,[32] the eventual outgrowth of MKK4-expressing cells is not due to a discrete genetic selection event. Rather, our data support a model in which the population of MKK4-expressing cells adapts to the consequences of MKK4 activation.

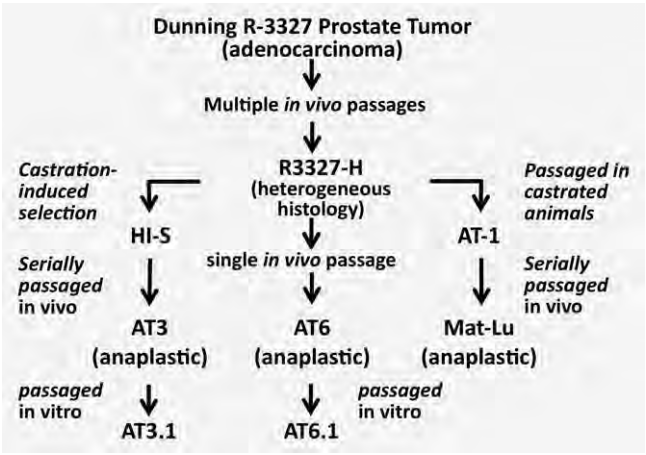

Figure 1. Summary of the derivation of the family of Dunning rat prostatic cancers used in this study. The Dunning rat prostate cancer lines include multiple cell lines of various histologies, aggressiveness and pattern of spread derived from a spontaneously originating prostate adenocarcinoma.

Material and Methods

Cell lines and culture conditions

AT6.1, AT3.1 and Mat-Lu Dunning rat prostate carcinoma cells were the generous gift of Dr. John Isaacs, The Johns Hopkins School of Medicine.[18,19] All cell lines, which have low endogenous levels of MKK4 relative to rat brain (positive control), tested mycoplasma negative with the Mycoplasma PCR ELISA per manufacturer's specifications (Roche Applied Science, Indianapolis, IN). Cells were maintained in standard media as described previously.[15] The construction of AT6.1-HA-MKK4 and AT6.1-pLNCX2 vector-only control cell lines has been reported previously.[15] The same methodology was used to derive AT3.1-HA-MKK4, Mat-Lu-HA-MKK4 and their corresponding pLNCX2 vector-only control cell lines. Clonal cell lines were established by limited dilution cloning and maintained in growth media containing G418.[15] Stable transfection of pmCherry into AT6.1 cell lines was similarly performed. First, the pmCherry expression cassette (Clontech) was subcloned into the pLHCX vector. Transfection of packaging cells with pLHCX-pmCherry constructs and retroviral infection of the target cancer cells were conducted as earlier. One representative AT6.1-HA-MKK4/Vector clone was engineered to also stably express pLHCX-pmCherry. Infected cells were selected and maintained in medium containing 400-μg/mL active Hygromycin B (Invitrogen) to select for stable pools. Stable pmCherrry expression was verified by fluorescence imaging and flow cytometry.

Establishment of metastasis-derived cell lines

At the experimental endpoint, lungs were harvested, PBS was injected through the trachea to inflate the lungs and individual surface metastases were removed, minced and placed in a 12 well plate with 1 mL of standard media containing 500-μg/mL active G418. Viable cells were cultured *in vitro* and expanded for immunoblotting and kinase assays.

Quantitation of disseminated AT6.1 cells in the lungs of mice

Primers and a probe (Integrated DNA Technologies, Coralville, Iowa) specific to an intronic portion of the rat β-globin gene were used to detect rat cells in mouse lung. The sequences were as follows: F: 5′-GTTTCAACATGAAGTAGAACAACAATATCA-3′, R: 5′-TCAGTAGTCATTCTGCCTGTCTTTTAA-3′, reporter probe: 5′-FAM-CACTGCAGGCCCATTTCAAATGGAG-3BHQ-1-3′. To normalize against mouse DNA, mouse-specific primers specific to an intronic portion of β-globin were also designed: F: 5′-GGCTGCCTGCCTTTAATTCA-3′, R: 5′-GGTTAGCTTGGATAACCTGCTTTTT-

3′, reporter probe: 5′-FAM-AGGGATTGTCCTGTCCTTC-CACGCTT-BHQ-1-3′. All DNAs were prepared with the PUREGENE DNA Purification Kit (Gentra Systems). DNA from each lung was purified into 200-μL Tris-EDTA and subsequently diluted 1:10 before adding 1 μL of DNA to the quantitative reverse transcription-PCR (qRT-PCR) reaction. Quantitative RT-PCR reactions were conducted as described.[13] All reactions were done in triplicate, with appropriate negative controls (100% mouse DNA for rat primers, 100% rat DNA for mouse primers, and water). To calculate the number of rat AT6.1 cells disseminated within the lung tissues, a standard curve was generated by qRT-PCR amplification for the rat β-globin gene from DNA isolated from resected lungs spiked *ex vivo* with various known numbers (1×10^2–1×10^7) of AT6.1 cells. The experimental amplification cycles were then compared with the standard curve to determine the number of AT6.1 cells in a given sample.

Immunoblotting and kinase assays

Monolayer cell cultures at 70–80% confluence were washed in ice-cold PBS, and protein lysates were prepared as previously described.[13,15] For protein isolated from metastases, individual metastases were rapidly washed in ice-cold PBS and homogenized using a 1-mL tissue grinder (Fisher Scientific) with 300 μL of the lysis buffer, after which the preparation steps were the same as for cells in culture. SDS-PAGE and immunoblotting were conducted as previously described.[15] The antibodies and dilutions are as follows: HA.11 (Covance) at 1:1,000 with antimouse secondary (Sigma) at 1:5,000; p38 (Cell Signaling) at 1:1,000 with antirabbbit (Cell Signaling) 1:5,000; and JNK (Cell Signaling) at 1:1,000 with antirabbit (Cell Signaling) 1:5,000. As a loading control, membranes were probed for actin (Calbiochem) at 1:5,000 followed by incubation with a goat antimouse secondary antibody (Calbiochem) at 1:20,000.

Kinase assays. Cell lines stimulated with 50 ng/μL anisomycin (Sigma) or unstimulated were scraped in 250-μL Triton X lysis buffer with protease inhibitors.[15] Tissues were washed in cold PBS and homogenized in 1.5-mL Triton X buffer with inhibitors. Tissue lysates were precleared by rotating the lysates overnight at 4°C with 30 μL of Protein A/G PLUS Agarose Beads (Santa Cruz Biotechnology, Santa Cruz, CA) and 0.667 μL per 100 μL of lysate of Normal Mouse IgG (Santa Cruz Biotechnology). Five hundred micrograms of cell lysate or 1.0 mg of tissue lysate were brought to a final volume of 600 μL with cold M2 immunoprecipitation buffer.[15] To this mixture, 4 μg (1:150) of 1 mg/mL of HA.11 antibody (Covance) and 30 μL of Protein A/G PLUS Agarose Beads were added. The mixture was rotated at 4°C for 16 hr. The beads were centrifuged and washed three times with ice-cold M2 buffer and three times with ice-cold 50 mmol/L HEPES (Media Tech). The washed beads were resuspended in 19 μL of cold double-distilled deionized water ($d3H_2O$) and mixed with 0.1-μg inactive recombinant p38α (Upstate); 5 μL of ATP mix [0.03 μL of magnesium/ATP mix (Upstate), 0.05 μL 10 mCi/mL γ-32P-ATP (Perkin Elmer), and 4.92 μL $d3H_2O$ per sample], and 5 μL of chilled 6X kinase reaction buffer[15] and 10 μM of p38 inhibitor SB03580 (Calbiochem). The kinase reactions were incubated at 30°C for 1 hr, with gentle mixing every 10 min. The reactions were spun down, and 20 μL of supernatant were withdrawn, and subjected to denaturing SDS-PAGE as described earlier. The gel was transferred to a nitrocellulose membrane and exposed to autoradiograph film, which was visualized for radioactively labeled p38 substrate at ∼36 hr. After detection of radiolabeled substrate, the membrane was immunoblotted for total p38 as a loading control.

Metastasis assays

All animal experiments are part of an Institutional Animal Care and Use Committee (IACUC)-approved protocol at The University of Chicago. All cell lines were maintained below passage 10, cultured for a minimum of two passages, and not grown beyond 80% confluence. Male six- to eight-week-old athymic nude mice (Harlan) were utilized for all animal experiments. Spontaneous metastasis assays were conducted as previously described.[15,16] For experimental metastasis assays, 1×10^4 cancer cells in 100-μL PBS were injected *via* lateral tail vein. Metastases were quantitated by visual inspection after the lungs were excised and inflated with 10% buffered formalin or cold PBS. Surface lung metastases (>1 mm^2 in size) were counted. For endpoint experiments, animals were followed closely for signs of advanced stage metastatic disease including weight loss, lethargy, breathing difficulty and euthanized according to standard IACUC protocols.

Evaluation of the cell cycle distribution of AT6.1-mcherry cells in the lung microenvironment

1×10^4 AT6.1-Vector or AT6.1-HA-MKK4 cells, which stably express pmCherry, were injected *via* tail vein as above. Mice were euthanized and their lungs harvested after perfusion with 1-mL cold PBS to wash out potentially autofluorescent erythrocytes.[33] The excised lungs were minced, placed in 5-mL DMEM with 8,000 units Collagenase Type III (Worthington Biochemical) and then incubated at 42°C for 45 min with rotational mixing. An additional 5-mL DMEM was added to the lung mixture, which was then dissociated using a Seward Stomacher 80 Biomaster (Brinkmann) on low for 15 min. The mix was filtered through a 48 μm nylon-mesh (Small Parts), and the filtrate centrifuged at 224*g* for 5 min. The cell pellet was resuspended in 5-mL DMEM. To prevent efflux of Hoechst dye out of the cells,[34] 50 μL of 50-mM Verapamil hydrochloride (Sigma) was added to the lung mixture and the suspension incubated at 37°C for 30 min with rotational mixing. The cells were then stained with 50 μL of 1 mg/mL Hoechst 33342 (Invitrogen) by incubation at 37°C for 45 min with rotational mixing. After staining, the mix was centrifuged at 224*g* for 5 min, and the cell pellet was resuspended in 2-mL PBS. Cell cycle analysis was performed *via* flow cytometry using a BD LSRII cytometer, and the analysis was

performed using FlowJo v.9.0.1. The entire volume of lung suspension was analyzed for each sample. Cellular aggregates and erythrocytes were excluded using a FSC *vs.* SCC plot. The pmCherry$^+$ population was determined using a 561-nm yellow-green laser with a 610/20 filter with fluorescence plotted *vs.* SSC. Cell-cycle data was collected using a 355-nm UV laser with a 450/50 filter plotted as a linear histogram. Because of relatively small populations, the pmCherry$^+$ cell-cycle distribution could not be directly determined. Therefore, the G_1, S and G_2/M gates were determined using the entire Hoechst stained population within each sample, transposed onto the mCherry$^+$ cell-cycle histogram and the percentage of pmCherry$^+$ cells within each gate was determined. Analysis of lungs spiked with 1×10^6 AT6.1 pmCherry$^+$ cells grown *in vitro* and processed as above served as the positive control and confirmed that the G_1, S and G_2/M gated populations for the entire lung corresponded to the respective populations of pmCherry$^+$ cells. Healthy lung tissues processed as above served as the negative control.

Statistical analyses

All statistical calculations were performed using SigmaPlot 11 (Systat Software) or STATA (Stata Corp, LP) in consultation with the biostatistics core at the University of Chicago. For surface lung metastasis comparison, the Mann-Whitney Rank Sum Test (*t* test) was used to compare cohorts. For overall survival, log-rank Kaplan–Meier analyses were performed. A nonlinear regression analysis was performed to determine the relationship between time and surface lung metastases. A modified, 2-parameter, exponential growth curve with the form $f(t) = \exp[b_0 + b_1{}^*(t - t_0)])$ was used to model the data. In this equation, $f(t)$ is equal to the number of measured surface metastases as a function of time, where t is the days post injection, t_0 represents the delay in days, b_0 is a calculated constant and b_1 is the exponential growth parameter. Finally, for cell cycle analyses, the percent of cells within a given phase of the cell cycle (G1, S and G2) was calculated as a percentage of the total cells within all three groups. The mean and standard error of the means for each animal cohort was calculated for each phase of the cell cycle, and a two-way analysis of variance (ANOVA) was performed to determine the potential interactions between days post injection and MKK4 status for each cell cycle phase.

Results

Ectopic MKK4 specifically suppresses the ability of highly metastatic cells to colonize target sites

Our laboratory previously identified MKK4 as a prostate cancer metastasis suppressor protein.[15,16] Optimization of the Dunning AT6.1 model system has enabled us to discern the time-ordered pattern of metastasis formation by AT6.1-Vector and AT6.1-HA-MKK4 cells using the spontaneous metastasis assay (Fig. 2*a*). Qualitative analyses of excised lung tissues (*e.g.*, histology and clonogenic assays) show that both AT6.1-Vector and AT6.1-HA-MKK4 tumor-bearing mice have disseminated cells and microscopic disease by 21 days post injection (dpi) of cancer cells subcutaneously suggesting that ectopic MKK4 impairs the formation of overt metastases after cancer cells lodge at the secondary site.[15] This implies that ectopic MKK4 should not have a significant effect on the number of AT6.1 cells lodging in the lungs. To test this assertion, the number of AT6.1-Vector and AT6.1-HA-MKK4 cells present in the lungs of tumor bearing mice at 21 dpi was quantitated using qRT-PCR for rat β-globin DNA. This assay showed that in both AT6.1-Vector and AT6.1-HA-MKK4 tumor-bearing animals had $\sim 1 \times 10^4$ cancer cells present in the lungs at 21 dpi (Fig. 2*b*). This finding is consistent with the observation that histological evaluation of specimens for both groups revealed no difference in the size or relative abundance of microscopic lesions by a pathologist (TL) (Fig. 2*c*).

The spontaneous metastasis model has been well validated as a means of interrogating the entire metastatic cascade, from tumor cell invasion through overt metastatic outgrowth. However, as tumor cells can be shed continuously from the primary tumor, cancer cell dissemination to the lungs may occur continuously over time, leading to potential heterogeneity of metastatic tumor biology at a given specific time point post injection. To directly test the ability of MKK4 to suppress metastatic colonization and to temporally synchronize lodging at the metastatic site, an experimental metastasis assay using 1×10^4 cancer cells injected into the blood stream directly *via* tail vein was used for subsequent experiments (Fig. 3*a*). Assays using three independent clonal AT6.1-Vector and AT6.1-HA-MKK4 cell lines showed that ectopic MKK4 caused an 87% reduction ($p < 0.001$) in metastatic colonization at 28 dpi (Figs. 3*b* and 3*c*). Specifically, the mean ($\pm$ standard error of the mean) number of overt surface lung tumors formed with AT6.1-Vector was 54.9 $\pm$ 6.0 as compared with 6.8 $\pm$ 1.6 for animals injected with AT6.1-HA-MKK4 cells. This decrease translated into a significant increase in survival with animals injected with AT6.1-HA-MKK4 cells showing a median survival of 42 dpi compared with 28 dpi for those injected with AT6.1-Vector cells [$p < 0.0001$; (Fig. 3*d*)]. Using spontaneous and experimental metastasis assays, it is therefore clear that ectopic expression of HA-MKK4 in AT6.1 prostate cancer specifically and powerfully inhibits metastasis formation through disruption of metastatic colonization.

Suppression of metastatic colonization by ectopic MKK4 expression is conserved across highly metastatic prostate cancer cell lines

The Dunning model is comprised of independent cell lines, which vary in histologies, hormonal dependence and metastatic abilities derived from a spontaneous rat prostate cancer (Fig. 1).[18,19] In particular, the Mat-Ly-Lu, Mat-Lu, AT3 and AT6 cell lines are independently derived highly metastatic variants.[19] To test the hypothesis that ectopic MKK4 expression suppresses metastatic colonization by more aggressive phenotypes than AT6.1, the Mat-Lu and AT3.1 cell lines

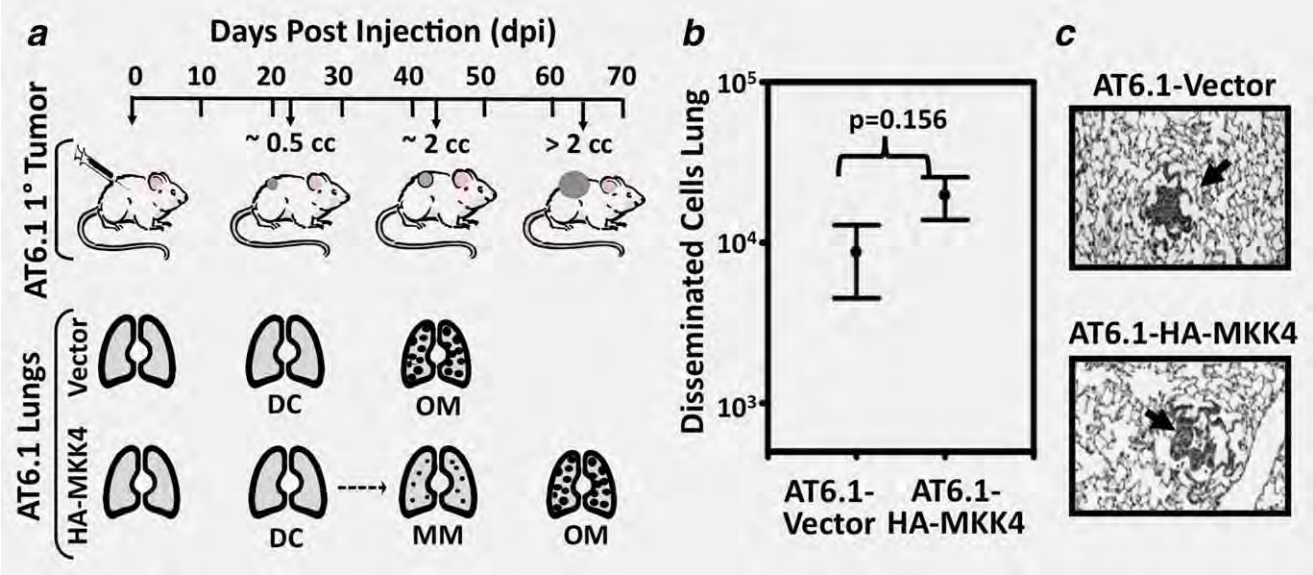

Figure 2. MKK4-mediated suppression of spontaneous metastasis formation is due to impaired metastatic colonization. (*a*) Schema for spontaneous metastasis assay. *Upper Panel* Injection of 2×10^5 AT6.1-Vector or AT6.1-HA-MKK4 cells subcutaneously into the flank of immunodeficient mice or syngeneic rats yields progressively growing tumors. *Lower Panel* The location and disposition of cancer cells at specific time points during spontaneous metastasis: disseminated cells (DC), microscopic metastases (MM), and overt metastases (OM) found in lungs at characteristic days post injection (dpi). (*b*) Quantitation of the number of disseminated cells within the lungs at 21 dpi using q-RT PCR. Data is shown as the mean ± standard error [*i.e.*, AT6.1-HA-MKK4 (8680 ± 4174 cells) *vs*. AT6.1-Vector (19684 ± 5842 cells) $p = 0.156$]. (*c*) Histologic appearance of microscopic metastases 21 dpi. Arrows denote foci of metastatic AT6.1-Vector and AT6.1-HA-MKK4 cells. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

were employed. Ectopic expression of HA-MKK4 was confirmed in independent, clonal Mat-Lu-HA-MKK4 and AT3.1-HA-MKK4 cell lines (Fig. 4*a left*). Since MKK4 can signal through both the p38 and JNK MAPKs, and to confirm that these signaling networks were intact, their expression was also confirmed by immunoblotting of both Vector- and HA-MKK4-expressing cell lysates (Fig. 4*a left*). To confirm that the ectopically expressed HA-MKK4 was functional, cells were activated with anisomycin, proteins were immunoprecipitated, and kinase activity was assayed (KA). *In vitro*, activated HA-MKK4 immunoprecipitated from cell lysates phosphorylated a purified p38 (GST-p38) substrate (Fig. 4*a right*). Results from experimental metastasis assays showed that ectopic MKK4 caused an 84% reduction in Mat-Lu metastatic colonization (Figs. 4*b* and 4*c*, $p = 0.045$). Animals injected with Mat-Lu-MKK4 cells also had a significant extension of median survival when compared with animals injected with Vector-only cell lines (Fig. 4*d*, $p < 0.0001$). Ectopic MKK4 similarly caused a dramatic (94%) reduction in AT3.1 colonization of the lung (Figs. 4*e* and 4*f*, $p < 0.001$) and also extended lifespan (Fig. 4*g*, $p < 0.0001$). Thus, the phenotype of suppression on metastatic colonization is generalizable to other, even more aggressive, Dunning prostate cancer cell lines. Experiments were thus undertaken, using the AT6.1 cell line as a representative model, to elaborate the details of how the suppression is imparted and overcome.

MKK4 specifically delays exponential growth of prostate cancer cells early after lodging within the lung

Ectopic MKK4 significantly reduces the number of overt experimental metastases and extends animal survival in both highly metastatic prostate and ovarian cancer xenograft models [Figs. 3 and 4 and Ref. 9]. Eventually, however, mice injected with HA-MKK4-expressing cells develop macroscopic metastases and succumb to their disease burden. This raises the important question of how disseminated cells may ultimately escape or bypass metastasis suppression. A long-standing paradigm of metastasis biology is that the process of metastasis selects for clones that have undergone permanent molecular changes (such as DNA mutation or deletion) enabling them to complete all steps of the metastatic cascade.[32] Studies were conducted to address the possibility that the outgrowth of MKK4-expressing prostate cancer metastases is due to such a canonical selective process through deletion or inactivation of the MKK4 kinase, or alternatively, that the eventual metastatic colonization is the result of a population-wide adaptation of cells to SAPK signaling. As a first step to discerning between these possibilities, the accumulation of overt AT6.1-Vector and AT6.1-HA-MKK4 experimental metastases over time was quantitated (Fig. 5*a*), with the hypothesis that similar to the ovarian cancer metastasis model,[13] AT6.1-HA-MKK4 and AT6.1-Vector lung colonization could be modeled with parallel exponential growth curves

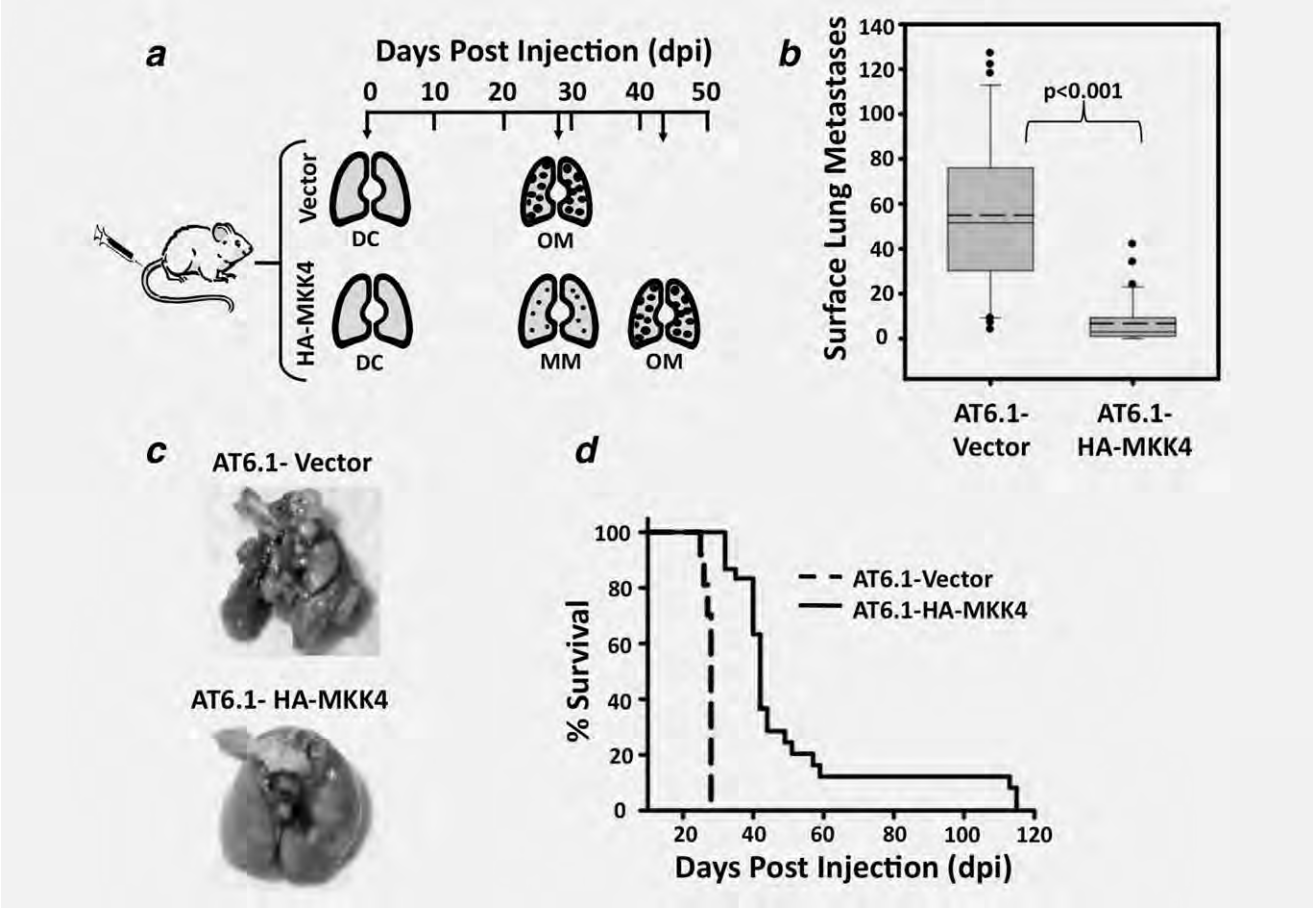

Figure 3. Ectopic expression of MKK4 suppresses metastatic colonization of AT6.1 prostate cancer cells in an experimental metastasis assay. (*a*) Schematic of the results from an experimental metastasis assay in which 1×10^4 cells are injected intravenously *via* tail vein venopuncture. (*b*) Box plot of the number of overt surface lung lesions present in mice injected with either AT6.1-Vector or AT6.1-HA-MKK4 cells *via* experimental metastases assay. Boundaries of box are 25th and 75th percentiles, with solid line indicating median, dashed line indicating mean, whiskers indicating 10th and 90th percentiles. The mean (± standard error of the mean) number of overt metastases AT6.1-Vector was 54.9 ± 6.0 as compared with 6.8 ± 1.6 for AT6.1-HA-cells ($p < 0.001$). (*c*) Representative gross pathology of lungs harvested from mice 28 dpi of either AT6.1-Vector or AT6.1-HA-MKK4 cells. (*d*) Kaplan–Meier survival analyses showed that the median survival for AT6.1 Vector animals was 28 days *vs*. 42 days for AT6.1-HA-MKK4 animals ($p < 0.0001$). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

indicating a transient delay in the initiation of metastatic colonization. The nonlinear regression model used to estimate the exponential growth rate and delay in growth imparted by HA-MKK4 expression was number of metastases $= \exp[b_0 + b_1{}^*(t - t_0)]$, as described in the methods above. In this equation, the b_0 parameter was calculated to be 0.5319, and b_1 was calculated at 0.1246. The adjusted R^2 of the model was 0.67. Tumor growth rates (b_1) were not statistically significantly different between HA-MKK4 and Vector mice ($p = 0.99$). Estimated tumor growth delay was 15.7 days (95% CI: 12.8–18.6) in the HA-MKK4 mice relative to vector. Thus, the HA-MKK4 growth curve was shifted in time, with metastatic colonization delayed by ~2 weeks (Fig. 5*a*).

On the basis of our findings, we hypothesized that outgrowth of HA-MKK4-expressing cells is due to an adaptive response of the population of cells and not a selection of cells that have deleted or inactivated the HA-MKK4 transgene. This hypothesis predicts that overt AT6.1-HA-MKK4 surface lung lesions will retain and express functional HA-MKK4. However, the protein will not be activated in overt metastases. Consistent with this hypothesis, biochemical analyses showed that ectopic HA-MKK4 is expressed in multiple, independent overt experimental metastases and metastasis-derived lines (MDLs) (Fig. 5*b*, *Upper* and *Lower Panels* respectively). However, MKK4 is not activated in these overt tumors as it does not phosphorylate GST-p38 in our *ex vivo* kinase assay [Fig. 5*c*, *Upper Panel* (KA)]. To rule out the possibility that ectopic HA-MKK4 in overt metastases has been rendered nonfunctional, AT6.1-HA-MKK4 MDLs were stimulated with anisomycin and used for *in vitro* kinase assays [Fig. 5*c*, *Lower Panel* (KA)]. HA-MKK4 is functional *in vitro* and phosphorylates GST-p38 substrate in a manner similar to parental cell line controls (positive control, *first lane*). As a loading control, the blot was also probed for the

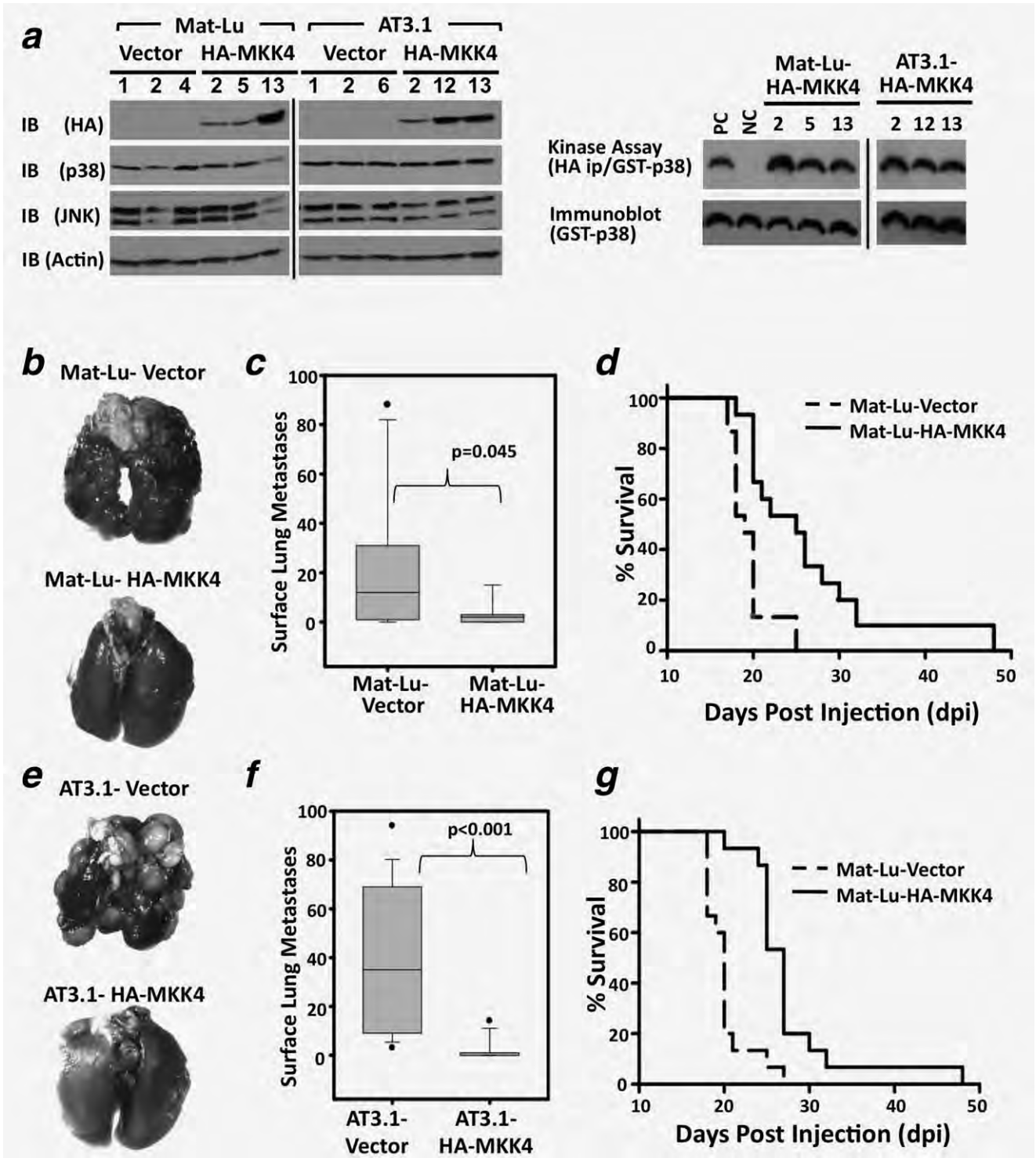

Figure 4. Ectopic HA-MKK4 specifically inhibits Mat-Lu and AT3.1 metastatic colonization. (*a*) Biochemical characterization of Mat-Lu-Vector, Mat-Lu-HA-MKK4, AT3.1-Vector and AT3.1-HA-MKK4 cell lines. *Left:* Lysates of three independent Vector and HA-MKK4 clones from each cell line were immunoblotted (IB) for the expression of a HA-tagged protein, p38, and JNK. Actin was used as a loading control. *Right*: Kinase assays (KA) showing functionality of ectopically expressed HA-MKK4. (*b*) Representative gross pathology of lungs harvested at 21 dpi from mice injected *via* tail vein with Mat-Lu-Vector and Mat-Lu-HA-MKK4 cells. (*c*) Box plot representation of the mean number ± standard error of the mean of overt surface experimental metastases formed by Mat-Lu-Vector was 25.3 ± 7.7 as compared with 4 ± 1.4 for Mat-Lu-HA-MKK4 cells ($p = 0.045$). (*d*) Kaplan–Meier survival analyses showed that the median survival for Mat-Lu-Vector animals was 19 days *vs*. 25 days for Mat-Lu-HA-MKK4 animals ($p < 0.0001$). (*e*) Representative gross pathology of lungs harvested at 21 dpi from mice injected with AT3.1-Vector and AT3.1-HA-MKK4 cells. (*f*) Box plot representation of the mean number ± standard error of the mean of overt surface metastases formed by AT3.1-Vector was 38.3 ± 7.6 as compared with 2.3 ± 1.1 for AT3.1-HA-MKK4 cells ($p < 0.001$). (*g*) Kaplan–Meier survival analyses showed that the median survival for AT3.1-Vector animals was 20 days *vs*. 27 days for AT3.1-HA-MKK4 animals ($p < 0.0001$).

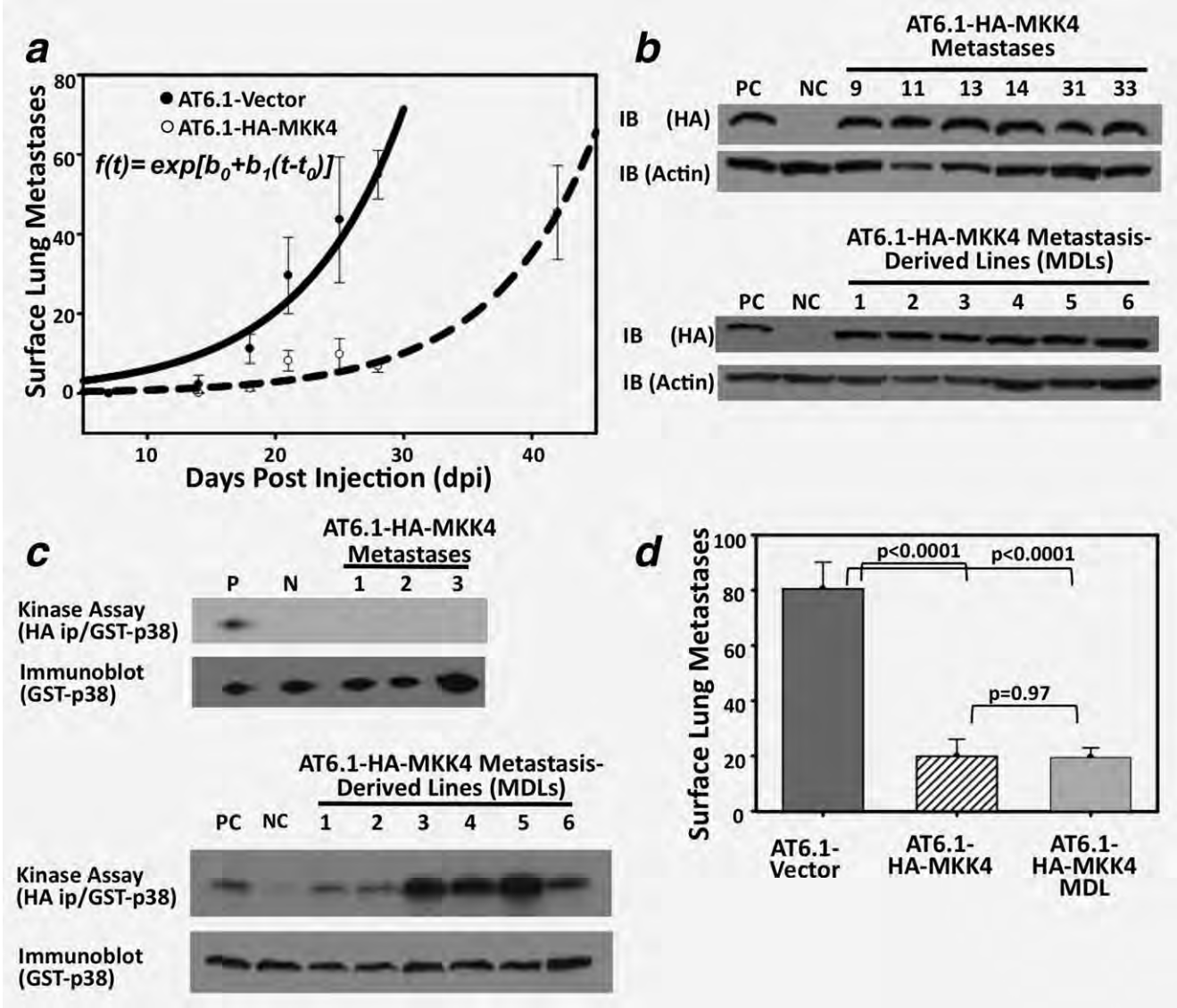

Figure 5. Transient suppression of metastatic colonization imparted by MKK4 not due to clonal loss or dysfunction of HA-MKK4 construct. (*a*) Nonlinear regression analysis of overt surface lung lesions as a function of time. Each point represents the mean ± SEM surface metastases from a minimum of five animals per time point. The adjusted $R^2 = 0.67$. The calculated t_0 delay for the AT6.1-HA-MKK4 curve is 15.7 days relative to the AT6.1-Vector curve. (*b*) Representative immunoblots (top) from>30 AT6.1-MKK4 overt experimental metastases and (bottom) from multiple independent metastasis derived cell lines (MDLs) demonstrating presence of HA-MKK4 construct within overt metastases and MDLs. (*c*) Representative *in vitro* kinase assay (top) AT6.1-MKK4 overt metastases and (bottom) from multiple independent metastasis derived cell lines (MDLs) showing inactive HA-MKK4 kinase within overt metastases; however HA-MKK4 remains functionally intact and activatable in cell lines derived from overt metastases. (*d*) Persistent suppression of metastatic colonization at 28 DPI with MDLs. The mean and SEM for surface metastases were 80.5 ± 9.7 for Vector, 19.8 ± 6.3 for HA-MKK4 parental and 19.4 ± 3.5 for HA-MKK4 MDLs ($p = 0.97$, MDL *vs*. parental AT6.1-vector $p < 0.0001$).

GST-p38 substrate (*bottom*). Similar results were observed with protein lysates and MDLs in the AT3.1 and Mat-Lu cell lines [data not shown].

Although AT6.1-HA-MKK4 MDLs express HA-MKK4, which can be artificially activated *in vitro*, it is possible that these cells have undergone other permanent molecular changes that will inactivate other aspects of the SAPK signaling pathway to compensate for ectopic HA-MKK4 expression. If a selection is indeed occurring, an experimental metastasis assay using the AT.61-HA-MKK4 MDLs should result in increased experimental metastasis formation compared with the parental HA-MKK4-expressing AT6.1 clones. To this end, metastasis-derived AT6.1-HA-MKK4 cell lines were assayed *in vivo* for the ability to suppress metastatic colonization after tail vein injection. Compared with vector-only cells, MKK4 MDLs displayed a significantly reduced ability to

form overt experimental metastases ($p < 0.001$ MDCL *vs.* Vector; Fig. 5*d*). Taken together, these *in vitro* and *in vivo* data strongly suggest that the eventual outgrowth of HA-MKK4-expressing cells is not due to selection for clones of cells that have permanently altered their MKK4 signaling status but is rather due to adaptation of the population to the biological consequences of SAPK signaling.

HA-MKK4-mediated suppression of metastatic colonization is concomitant with an accumulation of cells in G1 phase of the cell cycle

As shown in Figure 5*a*, metastasis suppression imparted by ectopic HA-MKK4 causes a delay ~14 day delay in metastatic colonization. Further, suppression is apparently homogeneous, with low variability in the number of overt metastases per lung at a given time point. Taken together, these data prompted the hypothesis that upon lodging, AT6.1-HA-MKK4 cells undergo a population-wide growth inhibition, perhaps *via* cell cycle arrest. To test this possibility, AT6.1-Vector and AT6.1 HA-MKK4 cells stably expressing mCherry protein were injected using the experimental metastasis assay, dissociated from the lung tissues at early time-points (7 and 14 days) post injection, and analyzed for DNA content using multiparameter flow cytometry analysis. Five animals per cell line and per time point were analyzed, and the number of cells analyzed and percentage of cells in each portion of the cell cycle is detailed (Fig. 6*a*) and graphically illustrated (Fig. 6*b*). There was no statistical difference in the number of cells analyzed in each cohort based on MKK4 status ($p = 0.80$) or days post injection ($p = 0.60$). A relative difference in the percentage of cells in the G1 and S phases of the cell cycle was noted in the HA-MKK4 expressing cells. There was a statistically significant increase in G1 cell cycle portion in AT6.1-HA-MKK4 cells compared with AT6.1-Vector controls ($p = 0.024$) with a concordant decrease in S phase fraction ($p = 0.037$). Taken together, these data depict a relative G1 arrest associated with ectopic MKK4 expression within the first 14 days upon metastatic dissemination, concordant with the delay in metastatic colonization.

Discussion

Accumulated evidence shows that many cancers including prostate cancer disseminate to metastatic sites early in the natural history of disease and can remain undetected and quiescent for extended periods of time.[3,4,10] In one recent report, disseminated prostate cancer cells (DTCs) can be detected in the bone marrow of 57% men who are without evidence of disease after prostatectomy. With a median follow-up of 42 months, 52% of those with DTCs have not recurred, including patients who still have DTCs 12 years after surgery.[5] Thus, in the context of prostate cancer recurrence, metastatic colonization, or the progressive outgrowth of disseminated cancer cells within a secondary site into clinically manifested metastases, is a particularly critical aspect to the multistep metastatic process.[7,35] The molecular factors that control the survival and eventual growth of these disseminated cells are largely unknown.

Experimental modulation of metastasis suppressor genes preclinically affords the unique opportunity to dissect the process of metastatic progression, specifically metastatic colonization, at the molecular level. This study shows that MKK4 has a powerful suppressive effect specifically on metastatic colonization in multiple highly metastatic prostate cancer cell lines, delaying the development of lethal metastases and significantly impacting survival. Prior work indicating that activation of the MKK4 kinase is critical for metastasis suppression,[15] along with data presented herein showing lack of MKK4 kinase activity within fully developed metastatic lesions, indicates that the alteration in biology within disseminated cells is critically tied to activation of the MKK4 kinase within the secondary microenvironment. Furthermore, the data on the kinetics of metastatic colonization are consistent with a populational dormancy associated with ectopic MKK4 expression in the immediate period after experimental dissemination. Adaptation and exponential metastatic colonization of the lungs by the MKK4 expressing cells following this period of suppression is eventual. Somewhat surprisingly, this adaptation was not due to a Darwinian permanent alteration in cancer cell biology promoting escape from suppression but rather seemed to be due to alteration in SAPK signaling over time. The "stress" leading to time-limited MKK4 kinase activation is unknown. Our results support the notion that metastatic colonization can be a rate-limiting step to metastatic progression, and that this process is dynamically regulated by complex interactions between disseminated cancer cells and the surrounding microenvironment. Current studies are pending using a novel *ex vivo* lung culture model[36] to test the hypothesis that there are microenvironmental factors (potentially including macrophages within the lung or factors expressed by the lung endothelium) regulating this process.

It is well established that prostate cancer in humans is a disease that disseminates to and most frequently colonizes the bones. Unfortunately, there are very limited preclinical prostate cancer models that metastasize to the bones and quantifying the metastatic progression to the bone is imprecise.[37] The Dunning model is to date the only spontaneously derived prostate cancer model with multiple independent cell lines that reproducibly metastasize.[37] As such, it is well suited for this study of metastatic colonization. Nonetheless, the effect of MKK4 on metastatic colonization of the bone microenvironment will be studied in future studies using human prostate cancer cell lines and genetically engineered mouse models that have an established pattern of bone metastasis.[37–39] Furthermore, interrogation of overtly metastastic tissue samples from rapid autopsy programs[40] and disseminated prostate cancer cells isolated using cutting edge techniques,[5,41] will be undertaken to explore the expression and activation of the SAPK pathway in metastatic patients samples.

a

	Median # analyzed (range)	%G1 (±SEM)*	%S (±SEM)**	%G2 (±SEM)
AT6.1-HA-MKK4 7DPI	155 (85-974)	69.0 (4.38)	6.4 (0.48)	24.6 (4.50)
AT6.1-Vector 7DPI	383 (98-4508)	53.3 (5.51)	15.3 (5.61)	31.4 (0.73)
AT6.1-HA-MKK4 14DPI	156 (64-352)	66.1 (4.29)	12.1 (1.81)	21.8 (4.1)
AT6.1-Vector 14DPI	100 (57-348)	55.2 (5.97)	19.23 (4.80)	25.6 (1.96)

* Increase in G1 fraction with HA-MKK4 (2-way ANOVA p=0.024)
** Decrease in S1 fraction with HA-MKK4 (2-way ANOVA p=0.037)

b

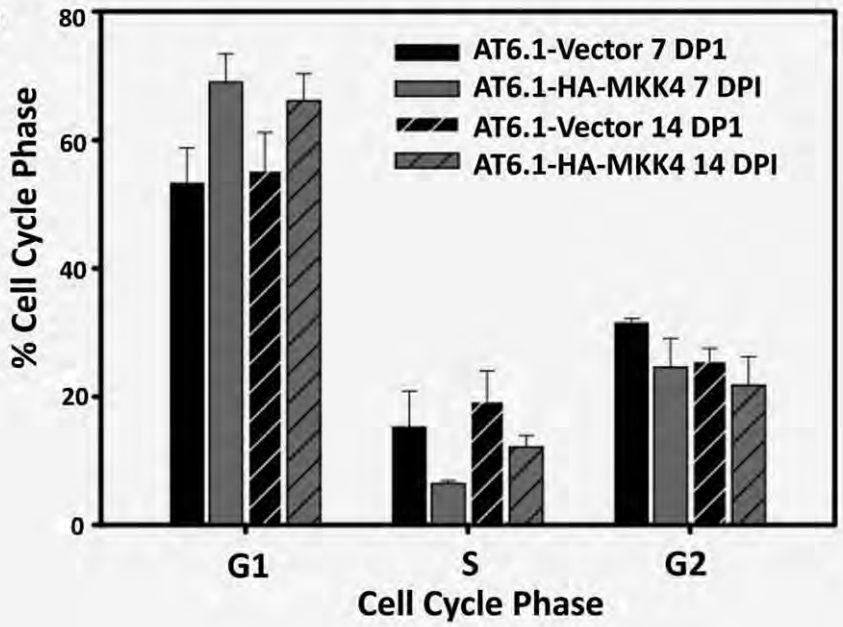

Figure 6. Ectopic expression of HA-MKK4 leads to a shift in cell cycle from S to G1 within disseminated AT6.1 prostate cancer cells. Disseminated AT6.1-Vector and AT6.1-HA-MKK4 cells were isolated from lungs at 7 and 14 days post injection and their cell cycle distribution was assessed *via* flow cytometry. (*a*) Tabular details of the number of cells analyzed and percentage of cells in each of the cell cycle. (*b*) Graphical representation of the cell cycle data. Bars represent the percentage of cells in each phase of the cell cycle with error bars representing standard error of the mean.

Optimization of methods to analyze the cell cycle distribution of relatively rare AT6.1-Vector, and AT6.1-HA-MKK4 cells lodged within the lung allowed the interrogation of these disseminated cancer cells with respect to their proliferative status. This study demonstrates that MKK4-expressing cells have a population shift in cell cycle from S phase to G1 phase within the first 7–14 dpi. In the parallel ovarian cancer metastasis mode, ectopic MKK4 expression seems to be associated with a decrease in cancer cell proliferation and an upregulation of the cell cycle regulator p21.[13] However, in that model, MKK4 signals through p38, whereas in the prostate model, it seems to signal through JNK.[11,15] Nonetheless, as this study supports a G1 cell cycle arrest as the mechanism for suppression of metastatic colonization, regulators of the G1 to S transition including p21, p27, p16 and cyclins D/E, represent potential direct or indirect targets of MKK4 activation that may be implicated as the mediators of MKK4 dependent metastasis suppression. Studies are currently underway using this prostate cancer experimental metastasis model and these flow cytometry techniques to elaborate the mechanistic underpinnings of the metastasis suppressor phenotype. Array-based expression studies of disseminated MKK4

expressing cancer cells could also identify inverse correlates of MKK4 expression, potentially negative regulators of this process, which could be targeted in an attempt to prolong the metastasis suppression.

Prostate cancer is a disease that can disseminate early, be variably quiescent over time, and which eventually completes the steps of the metastatic cascade to form lethal metastases. It is therefore essential to understand the precise biology that governs the kinetics of metastatic colonization. MKK4 appears to enable a shift from active to dormant cell cycling, thereby limiting proliferation of disseminated cells. As this process is transient and dependent on MKK4 activation, extension of kinase activation and/or MKK4 pathway activation may prolong suppression (*e.g.*, constitutively active kinase, pharmacologic activation of the kinase, inhibition of potential inverse correlates of MKK4 activation and specific activation of upstream effectors). As such, these and future studies have significant clinical implications for the design of therapies aimed at controlling disseminated tumor growth. Further work specifically testing this hypothesis that prolonged SAPK pathway activation can extend metastatic dormancy is necessary to enable future translational efforts targeting this process in micrometastatic prostate cancer patients.

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